Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary X-ray analysis of FliT, a bacterial flagellar substrate-specific export chaperone

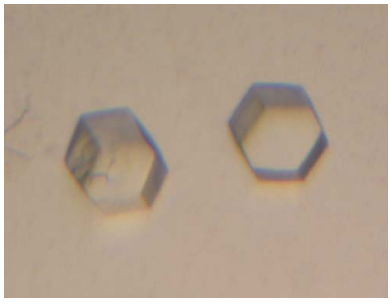
Miki Kinoshita,[a] Midori Yamane,[a]‡ Hideyuki Matsunami,[a,b] Tohru Minamino,[a,c] Keiichi Namba[a] and Katsumi Imada[a]*

[a]Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, [b]Trans-membrane Trafficking Unit, Okinawa Institute of Science and Technology, 12-22 Suzaki, Uruma, Okinawa 904-2234, Japan, and [c]PRESTO (Precursory Research for Embryonic Science and Technology), JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

‡ Present address: Graduate School of Material Science, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara, 630-0192, Japan.

Correspondence e-mail: kimada@fbs.osaka-u.ac.jp

Received 19 May 2009
Accepted 8 July 2009



The assembly process of the bacterial flagellum is coupled to flagellar gene expression. FliT acts not only as a flagellar type III substrate-specific export chaperone for the filament-capping protein FliD but also as a negative regulator that suppresses flagellar gene expression through its specific interaction with the master regulator $FlhD_4C_2$ complex. In this study, FliT of *Salmonella enterica* serovar Typhimurium was expressed, purified and crystallized. Crystals of SeMet FliT were obtained by the sitting-drop vapour-diffusion technique with potassium/sodium tartrate as the precipitant. The crystals grew in the trigonal space group $P3_121$ or $P3_221$ and diffracted to 3.2 Å resolution. The anomalous difference Patterson map of the SeMet FliT crystal showed significant peaks in its Harker sections, indicating the usefulness of the derivative data for structure determination.

1. Introduction

The bacterial flagellum is a huge protein complex composed of about 30 different proteins with copy numbers of between a few and a few tens of thousands (Macnab, 2003; Berg, 2003). Its construction is initiated by self-assembly of the basal body MS-C ring within the inner membrane. After completion of the MS-C ring, the axial protein subunits are delivered to the distal end through the central channel of the growing flagellar structure by the flagellar type III protein-export apparatus (Macnab, 2004; Minamino *et al.*, 2008). A remarkable feature of the flagellar assembly is that the export apparatus couples the assembly process to gene expression of flagellar proteins. The flagellar genes of *Salmonella enterica* serovar Typhimurium can be grouped into three classes (classes 1, 2 and 3) according to their transcriptional hierarchy (Kutsukake *et al.*, 1990). The class 1 promoter controls the expression of the flagellar master operon encoding FlhD and FlhC, which form a hetero-hexameric complex $FlhD_4C_2$ (Wang *et al.*, 2006). Together with a housekeeping sigma factor σ^{70}, $FlhD_4C_2$ activates gene expression of the class 2 operons (Liu & Matsumura, 1994), which encode the hook–basal body proteins and some of the regulatory factors, including the flagellar-specific transcription factor σ^{28} and the anti-σ^{28} factor FlgM. σ^{28} activates the class 3 promoters (Ohnishi *et al.*, 1992) encoding the late flagellar genes, including the filament subunit flagellin (Chilcott & Hughes, 2000). Until hook–basal body assembly is finished, FlgM remains in the cytoplasm and hence inhibits the activity of σ^{28} by forming a complex with σ^{28} to suppress class 3 gene expression. Upon completion of the hook structure at a length of about 55 nm, FlgM is secreted out of the cell, relieving this inhibition (Hughes *et al.*, 1993; Kutsukake, 1994). The tape-measure protein FliK transmits the hook-completion signal to the export apparatus and switches export specificity from rod/hook proteins to late flagellar proteins (Minamino *et al.*, 1999; Moriya *et al.*, 2006), allowing the complex macromolecular assembly to be built efficiently.

FliT is a 14 kDa protein composed of 122 amino-acid residues. Disruption of the *fliT* gene does not affect either flagellar assembly or motility, but increases the expression levels of the flagellar genes, indicating that FliT acts as a negative regulator of the class 2 genes

Table 1
Summary of the data statistics for SeMet FliT.

Values in parentheses are for the highest resolution shell.

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å, °)	$a = b = 121.3$, $c = 58.7$, $\gamma = 120$
Wavelength (Å)	0.97915
Resolution (Å)	42.2–3.2 (3.37–3.2)
Observations	32827 (4862)
Unique reflections	8390 (1219)
Completeness (%)	99.3 (99.9)
Redundancy	3.9 (4.0)
$\langle I/\sigma(I)\rangle$	11.1 (2.6)
$R_{\rm merge}$† (%)	10.1 (40.2)
$R_{\rm anom}$‡ (%)	8.5 (28.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl. ‡ $R_{\rm anom} = \sum_{hkl} |\langle I(hkl+)\rangle - \langle I(hkl-)\rangle| / \sum_{hkl}[\langle I(hkl+)\rangle + \langle I(hkl-)\rangle]$, where $\langle I(hkl+)\rangle$ and $\langle I(hkl-)\rangle$ correspond to the average intensities of each Friedel pair for reflection hkl.

(Yokoseki *et al.*, 1995). FliT binds to the $FlhD_4C_2$ complex and prevents it from activating class 2 promoters (Yamamoto & Kutsukake, 2006). Since FliT binds to the filament-capping protein FliD and facilitates the export of FliD (Fraser *et al.*, 1999), FliT is thought to be the export chaperone for FliD (Evans *et al.*, 2006). Moreover, FliT can bind to FliJ, a cytoplasmic component of the flagellar type III export apparatus (Evans *et al.*, 2006; Minamino *et al.*, 2000). Thus, FliT seems to play an important role in fine-tuning the flagellar-assembly process. In order to understand the molecular mechanisms of flagellar gene regulation and the export process, the structure of FliT is essential. Here, we report the expression, purification and preliminary X-ray crystallographic studies of FliT.

2. Materials and methods

2.1. Protein expression and purification

A *Nde*I–*Bam*HI fragment encoding the *fliT* gene of *S. enterica* serovar Typhimurium (GenBank accession No. M85241) was inserted into the *Nde*I–*Bam*HI site of pET3c (Novagen) to create plasmid pHMK213. A 30 ml overnight culture of BL21(DE3)pLysS carrying pHMK213 was inoculated into 3 l LB medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre) containing 50 µg ml^{-1} ampicillin and 30 µg ml^{-1} chloramphenicol. Cells were grown at 310 K until the culture density reached an OD_{600} of 0.5. Expression of full-length FliT was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 m*M* and the culture was continued for 16 h at 299 K. The cells were harvested by centrifugation (7900*g*, 10 min, 277 K) and stored at 193 K. The cells were thawed, suspended in 50 m*M* Tris–HCl pH 7.8, 1 m*M* EDTA with a tablet of Complete protease-inhibitor cocktail (Boehringer Mannheim) and sonicated (Astrason model XL2020 sonicator, Misonix Inc.). The cell lysate was centrifuged (145 000*g*, 10 min, 277 K) to remove cell debris. The supernatant was loaded onto a DEAE Sepharose column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0 and proteins were eluted using a linear gradient of 0–1 *M* NaCl. Fractions containing FliT were precipitated by the addition of acetic acid until the pH value reached 4.5 and were stored at 277 K for 20 min. The precipitant was collected by centrifugation (125 000*g*, 10 min, 277 K) and dissolved in 20 m*M* sodium citrate pH 4.0 and 6 *M* urea. After removal of the insoluble material by centrifugation (145 000*g*, 10 min, 277 K), the protein solution was applied onto a Hi-Load SP-Sepharose HP column (GE Healthcare) equilibrated with 20 m*M* MES–NaOH pH 6.0, 6 *M* urea and was eluted with a linear gradient of 0–400 m*M* NaCl. The peak fractions were dialyzed overnight against 10 m*M* Tris–HCl pH 8.0, concentrated and loaded onto a Hi-Load Superdex 75 (26/60) column (GE Healthcare) equilibrated with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl. Fractions containing FliT were collected and dialyzed overnight against 10 m*M* Tris–HCl pH 8.0. The purity of the product was examined by SDS–PAGE and MALDI–TOF mass spectrometry (Voyager DE/PRO, Applied Biosystems).

Since FliT contains four methionine residues in its 122-amino-acid sequence, we prepared SeMet-labelled FliT for phase determination. SeMet FliT was prepared from the same strain used for the expression of native FliT as described by Guerrero *et al.* (2001). Cells cultured overnight in 200 ml LB medium at 310 K were harvested by centrifugation (5000*g*, 5 min, 277 K), washed and suspended in 0.9% NaCl solution. The cells were inoculated into 1 l of a minimal medium [a 1 l solution containing 1 g ammonium chloride, 3 g KH_2PO_4 and 7.73 g $Na_2HPO_4{\cdot}12H_2O$ was autoclaved and mixed with a 100 ml solution containing 20 g glucose, 0.3 g $MgSO_4$, 10 mg $Fe_2(SO_4)_3$ and 10 mg thiamine] containing 50 µg ml^{-1} ampicillin and 30 µg ml^{-1} chloramphenicol and cultured at 310 K until the culture density reached an OD_{600} of 0.7. L-Selenomethionine and IPTG were then added to final concentrations of 50 µg ml^{-1} and 1 m*M*, respectively, and the culture was continued for 16 h at 299 K. The cells were harvested and stored as for native FliT. In contrast to native FliT, most SeMet FliT was expressed as insoluble products (data not shown). Therefore, the cells were sonicated in 50 m*M* Tris–HCl pH 7.8, 1 m*M* EDTA, 6 *M* urea and the homogenate was centrifuged (7800*g*, 5 min, 277 K) to remove cell debris. Acetic acid was then added to the supernatant to a final pH value of 4.5 and the supernatant was stored at 277 K for 20 min. After centrifugation (7800*g*, 5 min, 277 K), the supernatant was loaded onto a HiTrap SP HP column (GE Healthcare) equilibrated with 20 m*M* MES–NaOH pH 6.0, 6 *M* urea and the proteins were eluted with a linear gradient of 0–400 m*M* NaCl. The fractions containing SeMet FliT were dialyzed overnight against 20 m*M* Tris–HCl pH 8.0, applied onto a HiTrap Q HP column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0 and proteins were eluted with a linear gradient of 0–400 m*M* NaCl. The peak fractions containing SeMet FliT were dialyzed against 10 m*M* Tris–HCl pH 8.0, concentrated and loaded onto a Hi-Load Superdex 75 (26/60) column using the same protocol as for native FliT. The incorporation of SeMet into FliT was examined and confirmed by MALDI–TOF mass spectrometry (Voyager DE/PRO, Applied Biosystems).

2.2. Crystallization

Initial crystallization screening of FliT was conducted at 277 and 293 K by the sitting-drop vapour-diffusion technique using Wizard I and II screening kits (Emerald BioStructures) and Crystal Screens I and II (Hampton Research). Each drop was prepared by mixing 1 µl protein solution (2.2–2.4 mg ml^{-1} FliT, 10 m*M* Tris–HCl pH 8.0) with 1 µl reservoir solution and was equilibrated against 170 µl reservoir solution. The crystallization conditions were optimized by varying the precipitant concentration, pH and additives using the sitting-drop method with the same drop preparation as for the initial screening or the hanging-drop method with the same drop preparation except for the reservoir volume (1 ml).

Since the solubility of SeMet FliT was much greater than that of native FliT, primary crystallization screening using Wizard I and II and Cryo I and II screening kits (Emerald BioStructures) and Crystal Screens I and II was carried out for SeMet FliT in addition to screening around those conditions in which native crystals were

grown. Drops were prepared by mixing 0.5 µl protein solution (2.4–10.8 mg ml^{-1} SeMet FliT, 10 m*M* Tris–HCl pH 8.0) with 0.5 µl reservoir solution and were equilibrated against 90 µl reservoir solution for the sitting-drop method and 1 ml for the hanging-drop method. The sitting-drop plates were prepared using a crystallization robot (Phoenix, Art Robbins Instruments).

2.3. Data collection and processing

X-ray diffraction data were collected on SPring-8 beamline BL41XU (Harima, Japan). Crystals were soaked in a solution containing 90%(*v*/*v*) reservoir solution and 10%(*v*/*v*) MPD for a few seconds, immediately transferred into liquid nitrogen for freezing and mounted on a goniometer in a cryo-gas flow. The diffraction data were recorded on an ADSC Quantum 315 CCD detector (Area Detector Systems Corporation) at 35 K using a helium cooling device (Rigaku) to reduce the radiation damage. The crystal-to-detector distance and the oscillation range were set to 300 mm and 1°, respectively. Diffraction spots were indexed and the intensities were integrated and scaled using the programs *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Statistics for the data collection are summarized in Table 1.

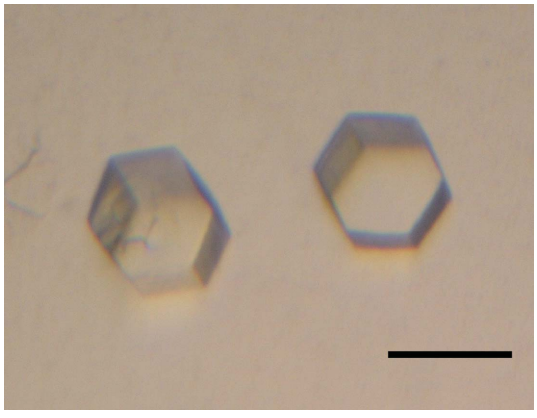


Figure 1
Hexagonal plate-shaped crystals of SeMet FliT. The scale bar is 0.1 mm in length.

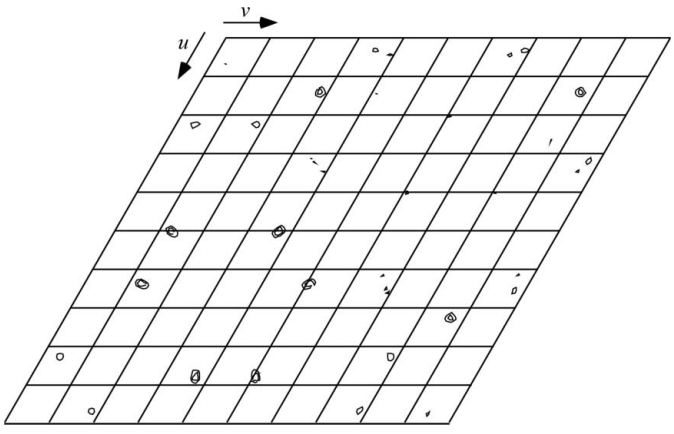


Figure 2
Bijvoet difference Patterson map at w = 0.33 (Harker section). The map was calculated using the data from the SeMet derivative at 4.0 Å resolution. The contour lines are drawn from 2.0σ to 4.0σ with an increment of 0.5σ.

3. Results and discussion

After four months of incubation at 277 K, tiny hexagonal plate-shaped or rod-shaped crystals of FliT appeared in a drop containing 1,6-hexanediol and $MgCl_2$ at pH 8.5 (Crystal Screen II, No. 39). We optimized the conditions and finally obtained hexagonal plate-shaped crystals with typical dimensions of 0.1 × 0.1 × 0.02 mm from drops prepared by mixing 1 µl protein solution (2.4–2.8 mg ml^{-1}) with 1 µl reservoir solution containing 0.1 *M* Tris–HCl pH 8.5, 2.8–3.4 *M* 1,6-hexanediol and 25–100 m*M* $MgCl_2$ at 277 K after six weeks of incubation. SeMet FliT crystals were also grown from the same reservoir conditions as used for native FliT. The crystals diffracted to 2.7 Å resolution and the space group was trigonal $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 118.5$, $c = 80.9$ Å. Unfortunately, analysis of the intensity statistics using the program *TRUNCATE* (Collaborative Computational Project, Number 4, 1994) indicated that the crystals were almost perfectly twinned. At this stage, we found that SeMet FliT remained soluble at a protein concentration of as high as 11 mg ml^{-1}, while native FliT tended to form aggregates when concentrated to greater than 2.8 mg ml^{-1}. Therefore, we restarted screening for a broader condition with the screening kits using a highly concentrated SeMet FliT solution (10.8 mg ml^{-1}).

Within two weeks, hexagonal plate-shaped crystals (Fig. 1) were grown in a drop containing 0.1 *M* CHES–NaOH pH 9.5, 1 *M* potassium/sodium tartrate and 200 m*M* $LiSO_4$ (Wizard I, No. 38) at 277 K. No crystals of native FliT were obtained around this reservoir condition, probably owing to the low protein concentration. The crystals diffracted to 3.2 Å resolution and belonged to the trigonal space group $P3_121$ or $P3_221$. The shape and the size of the crystals were almost same as those of the twinned crystals, but the unit-cell parameters were different: $a = b = 121.3$, $c = 58.7$ Å. The Matthews coefficient (V_M; Matthews, 1968) suggested the presence of two to four FliT molecules in the asymmetric unit, with a solvent content of between 73 and 47%. The self-rotation function map calculated using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) indicated the presence of a twofold noncrystallographic symmetry axis near the crystallographic *ab* plane. No other significant peaks were observed.

The Bijvoet difference Patterson map of SeMet FliT showed significant clear peaks on the Harker sections (Fig. 2), suggesting the usefulness of these data for phasing by the SAD method.

We thank N. Shimizu, M. Kawamoto and K. Hasegawa at SPring-8 for technical help in the use of beamline BL41XU. This work was supported in part by Grants-in-Aid for Scientific Research (18074006 to KI and 16087207 to KN) and the Targeted Proteins Research Program (TPRP) from the Ministry of Education, Science and Culture of Japan.

References

Berg, H. C. (2003). *Annu. Rev. Biochem.* **72**, 19–54.
Chilcott, G. S. & Hughes, K. T. (2000). *Microbiol. Mol. Biol. Rev.* **64**, 694–708.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Evans, L. D. B., Stafford, G. P., Ahmed, S., Fraser, G. M. & Hughes, C. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 17474–17479.
Fraser, G. M., Bennett, J. C. & Hughes, C. (1999). *Mol. Microbiol.* **32**, 569–580.
Guerrero, S. A., Hecht, H.-J., Hofmann, B., Biebl, H. & Singh, M. (2001). *Appl. Microbiol. Biotechnol.* **56**, 718–723.
Hughes, K. T., Gillen, K. L., Semon, M. J. & Karlinsey, J. E. (1993). *Science*, **262**, 1277–1280.
Kutsukake, K. (1994). *Mol. Gen. Genet.*, **243**, 605–612.
Kutsukake, K., Ohya, Y. & Iino, T. (1990). *J. Bacteriol.* **172**, 741–747.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.

Liu, X. & Matsumura, P. (1994). *J. Bacteriol.* **176**, 7345–7351.
Macnab, R. M. (2003). *Annu. Rev. Microbiol.* **57**, 77–100.
Macnab, R. M. (2004). *Biochim. Biophys. Acta*, **1694**, 207–217.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Minamino, T., Chu, R., Yamaguchi, S. & Macnab, R. M. (2000). *J. Bacteriol.* **182**, 4207–4215.
Minamino, T., González-Pedrajo, B., Yamaguchi, K., Aizawa, S. I. & Macnab, R. M. (1999). *Mol. Microbiol.* **34**, 295–304.
Minamino, T., Imada, K. & Namba, K. (2008). *Mol. Biosyst.* **4**, 1105–1115.
Moriya, N., Minamino, T., Hughes, K. T., Macnab, R. M. & Namba, K. (2006). *J. Mol. Biol.* **359**, 466–477.
Ohnishi, K., Kutsukake, K., Suzuki, H. & Iino, T. (1992). *Mol. Microbiol.* **6**, 3149–3157.
Wang, S., Fleming, R. T., Westbrook, E. M., Matsumura, P. & McKay, D. B. (2006). *J. Mol. Biol.* **355**, 798–808.
Yamamoto, S. & Kutsukake, K. (2006). *J. Bacteriol.* **188**, 6703–6708.
Yokoseki, T., Kutsukake, K., Ohnishi, K. & Iino, T. (1995). *Microbiology*, **141**, 1715–1722.